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TITLE: Acquired Tamoxifen Resistance and Overexpression of Anti-Apoptotic Molecules: A Potential Strategy for Overcoming Endocrine Resistance

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13. ABSTRACT (Maximum 200 Words)

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the chemo- and endocrine-resistance in breast cancer. We have investigated the *in vitro* anti-tumor activity of (-)-gossypol, a potent small molecule inhibitor of Bcl-xL, and the potential synergistic effects of (-)-gossypol in combination with chemodrugs and Tamoxifen in breast cancer cell lines. (-)-gossypol showed potent anti-tumor activity to human breast cancer cell lines with high levels of Bcl-xL, but has only minimal effect on human normal breast epithelial cells with low Bcl-xL. (-)-gossypol potently enhanced growth inhibition by doxorubicin and docetaxel, currently used chemotherapeutic agents for breast cancer, both *in vitro* and *in vivo*. However, (-)-gossypol did not show significant enhancement of Tamoxifen activity in ER(+) breast cancer MCF-7 and T47D cells. Bcl-xL knockdown by siRNA abolished the tumorigenecity of MCF-7 cells. The data support that Bcl-xL plays a critical role in breast cancer initiation, progression and chemoresistance, but its role in endocrine resistance remains to be further elucidated. The study provide us a solid foundation to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression.

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I. Introduction:

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the chemo- and endocrine-resistance of breast cancer. Our hypothesis is that the anti-apoptotic molecule Bcl-xL may play certain role in apoptosis resistance as well as in acquired Tamoxifen resistance, and a small molecule Bcl-xL inhibitor might be able to block the development of this resistance and increase the effectiveness of chemo/hormone therapy.

Our basic hypothesis to be tested is that Bcl-xL is the primary molecular target that mediate the anticancer activity of the small molecule Bcl-xL inhibitor (-)-gossypol in human breast cancer cells. Our ultimate goal is to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression. In this project, we will investigate *in vitro* and *in vivo* anti-tumor activity and the mechanism of action of (-)-gossypol in human breast cancer with Bcl-xL overexpression, and investigate the potential synergistic effects of (-)-gossypol in combination with chemotherapy and hormone therapy.

II. Key research accomplishments:

This project is one-year Concept Award project. Due to the time required to finish the animal study, a 6-month no-cost extension was requested and approved. During the project period, we carried out the tasks proposed in the Statement of Work. Specifically, we carried out the following studied:

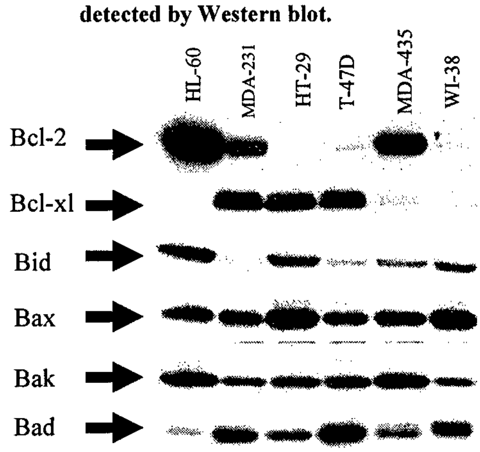
II.1. To analyze the correlation of the expression levels of Bcl-xL and response to tamoxifen, to assess whether there is any link between Bcl-xL and antiestrogen response. *(Task 1)*

II.1.1. Using human breast cancer cell lines with different levels of Bcl-xL to compare their cellular responses to antiestrogen therapy.

MCF-7 and T47D cells are ER(+) breast cancer cell lines and have high levels of Bcl-xL (Figure 1). MCF-7 and T47D cells responded to anti-estrogen therapeutics, Tamoxifen and Faslodex (ICI 182780, or ICI), in a dose-dependent manner (Figure 2). In our hands, the estradiol (E2) doses from 1×10^{-9} M to 1×10^{-10} M gave the optimal cell growth stimulation in these cells (Figure 2), and E2 1×10^{-10} M dose was chosen for the subsequent experiments.

The time course experiment in Figure 2 indicated that culture for 7 days is the optimal time point for E2-mediated cell growth. At this condition, both Tamoxifen and ICI dose-dependently inhibited the cell growth induced by E2 (Figure 2).

Figure 1. Expression status of Bcl-2 family of proteins in 6 representative cell lines as detected by Western blot.



II.1.2. Using existing breast cancer tissues from the tissue bank in our Cancer Center Breast Cancer Program to assess ER and Bcl-xl expression and correlation with clinical response to antiestrogen therapy.

In collaboration with University of Michigan Comprehensive Cancer Center Pathology and Immunohistology Core, we tried to perform immunohistochemistry (IHC) staining of tumor tissues to assess Bcl-xL expression. We have tested various Bcl-xL antibodies reportedly suitable for IHC from three vendors, the IHC staining we obtained were not distinctive enough to give us a convincing score for evaluation, although the histologists in the Core are well-trained and have many years' experience with IHC for breast cancer tissues. We are currently testing more antibodies, and using Bcl-xL-gene transfected tumor cell xenografts as positive control. However, within the funded Concept Award period, we did not obtain convincing IHC data to draw any conclusions.

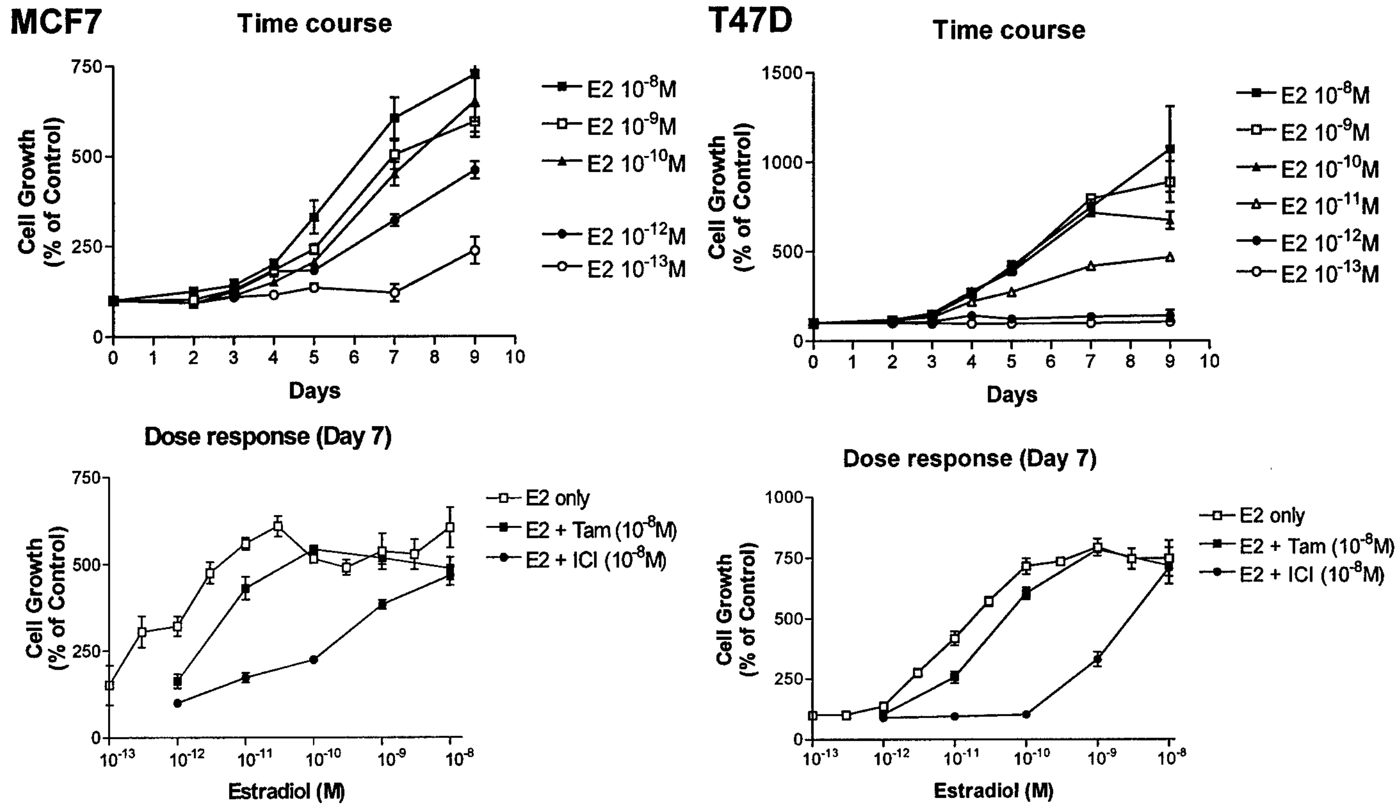


Figure 2. MCF7 and T47D cells in 96-well plates (4000/well) were treated with E2 or E2 plus antagonist, at serial concentrations. The cell growth was measured by MTT-based WST-1 cell counting kit and presented as Percent of Vehicle Control. Cells were cultured in phenol red-free IMEM + 5% CSS. (n=3-6)

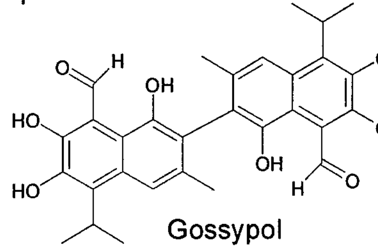
II.2. To investigate the effect of combining chemo/hormone therapy with Bcl-xL inhibitor, to assess whether Bcl-xL inhibitor can delay or prevent the development of chemo/hormone resistance. *(Task 2)*

II.2.1. *Using MCF-7 and T47D cells to investigate whether a Bcl-xl inhibitor can enhance the anti-tumor activity of chemo/hormone therapy.*

II.2.1.(a) Small molecule inhibitor of Bcl-xL

Through structure-based design, we and others have discovered a natural product from cottonseed, **gossypol**, to be the most potent small molecule inhibitor of Bcl-xL(1, 2). The chemical structure of gossypol is shown in **Chart I**. Gossypol binds to the BH3 (Bcl-2 homology domain 3) binding pocket of Bcl-xL which is essential for its anti-apoptotic function. Gossypol induces apoptosis in cancer cells with a high level of Bcl-xL protein, but has minimal effect on cancer or normal cells with low Bcl-xL and low Bcl-2. Gossypol has two enantiomer forms, i.e., (-)-gossypol and (+)-gossypol. Previous studies on gossypol for its anticancer activity have been performed exclusively on (±)-gossypol. Our recent data suggest that (-)-gossypol is the active form mediating its anti-tumor activity. In this project, we focused on (-)-gossypol in our studies.

Chart I. Chemical structure of gossypol, a potent Bcl-xL inhibitor



Gossypol

To provide a further insight on the structural basis of the binding between to gossypol and Bcl-xL, we have determined the three-dimensional structure of gossypol in complex with Bcl-xL using multi-dimensional NMR methods. The 3D NMR complex structure conclusively shows that gossypol binds to the BH3 binding site in Bcl-xL, where Bak or Bad BH3 peptide binds (**Figure 3**).

Analysis of this 3D structure showed that gossypol optimally interacts with Bcl-xL.

Using a quantitative fluorescence polarization-based (FP) binding assay, we have determined that gossypol potently inhibits the binding of Bcl-xL to Bak BH3 peptide with a K_i value of 0.6 μM, similar to that of Bak protein (**Figure 4**). Gossypol also moderately inhibits the binding of Bcl-2 to Bak BH3 peptide with K_i value of 10 μM (data not shown).

Figure 4. Binding of gossypol to Bcl-X_L as determined by FP-based binding assay.

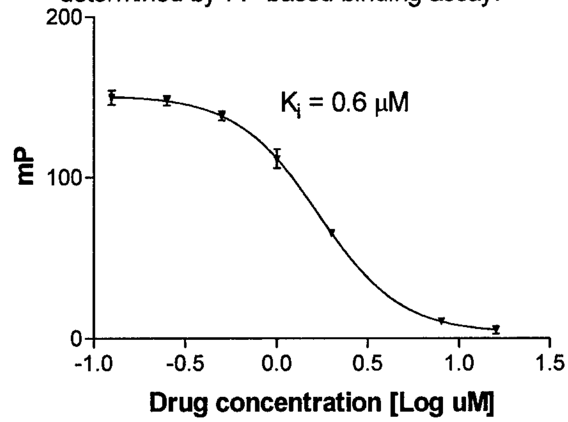
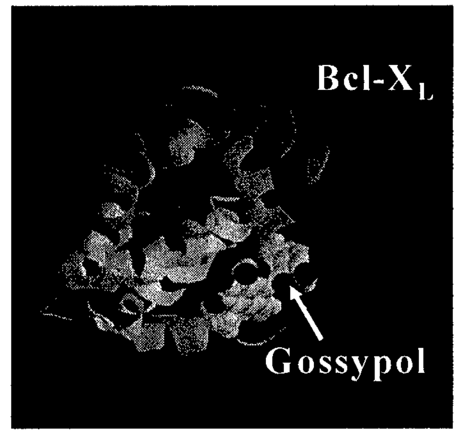


Figure 3. Experimental NMR solution structure of gossypol in complex with Bcl-X_L



II.2.1.(b) Gossypol mediated chemosensitization

To evaluate whether gossypol can enhance the anti-tumor activity of chemo/hormone therapy, we employed the Chou-Talalay combination index-isobologram and multiple drug dose-effect analysis method as described(3). Briefly, in 96-well plates, 3000 - 5000 cells/well human breast cancer cells were treated with (-)-gossypol and chemo/hormone therapeutic agents, alone or in combination. 4-6 days later, the cell survival was determined by WST-1 cell counting kit (Roche). The combination Index (CI) and Dose Reduction Index (DRI) for each combination dose ratio was calculated using the CalcuSyn software (www.biosoft.com). The optimal dose ratio in the drug combination that gives maximum synergy will be established based upon CI and DRI.(4)

Figure 5 shows the results of gossypol in combination with doxorubicin (DOX) and paclitaxel (Taxol) in MCF-7 cells. The combination index (CI) less than 1 indicates a more than additive effect, or synergy, in the drug combination. As shown in **Figure 5**, gossypol showed synergism in combination with DOX (CI = 0.56) and Taxol (CI = 0.24). Similar results were also observed in T47D cells. The **Table 1** summarizes the combination data in both MCF-7 and

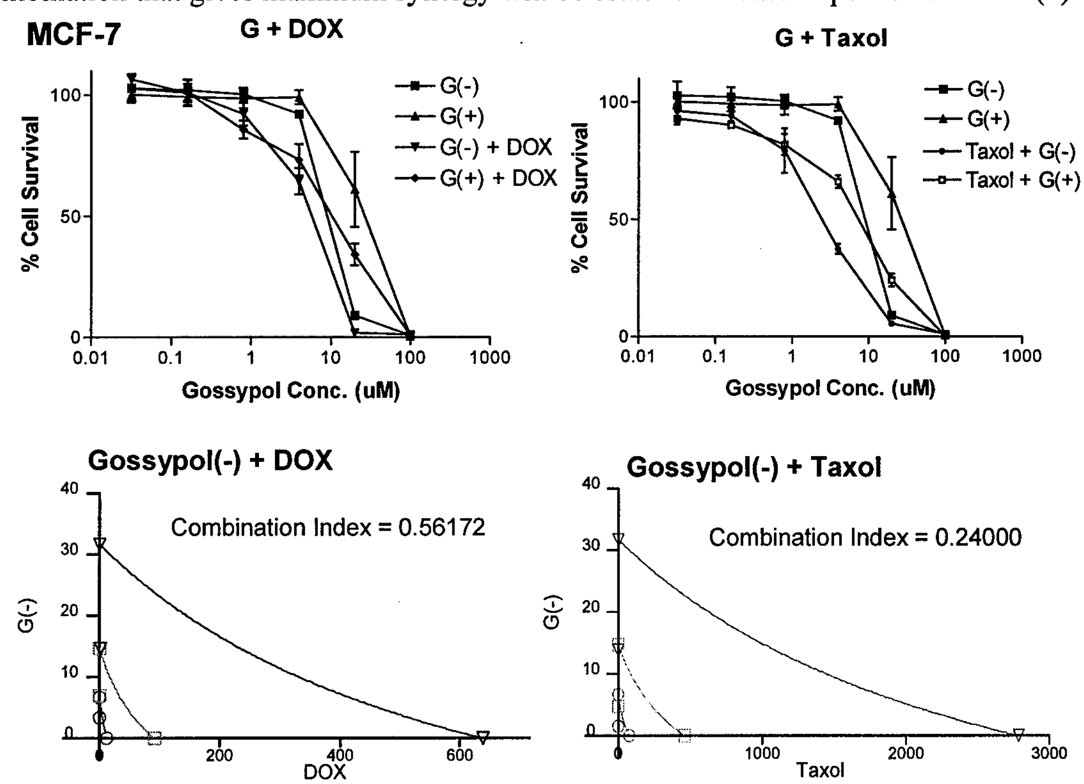


Figure 5. Gossypol enhances cytotoxicity of chemotherapeutic agents in MCF-7 cells. The lower two graphs are isobolograms corresponding to the WST assay data above. (n=3)

T47D cells. From **Table 1**, we conclude that (-)-gossypol have better anti-tumor activity and more synergy than (+)-gossypol, in combination with chemotherapeutic agents, DOX, Taxol and gemcitabine (Gem). Gossypol showed minimal toxicity to MCF-10A cells, the transformed human mammary epithelial cell line which has low levels of Bcl-xL (data not shown).

Table 1. Summary of drug combination data in MCF-7 and T47D cells.

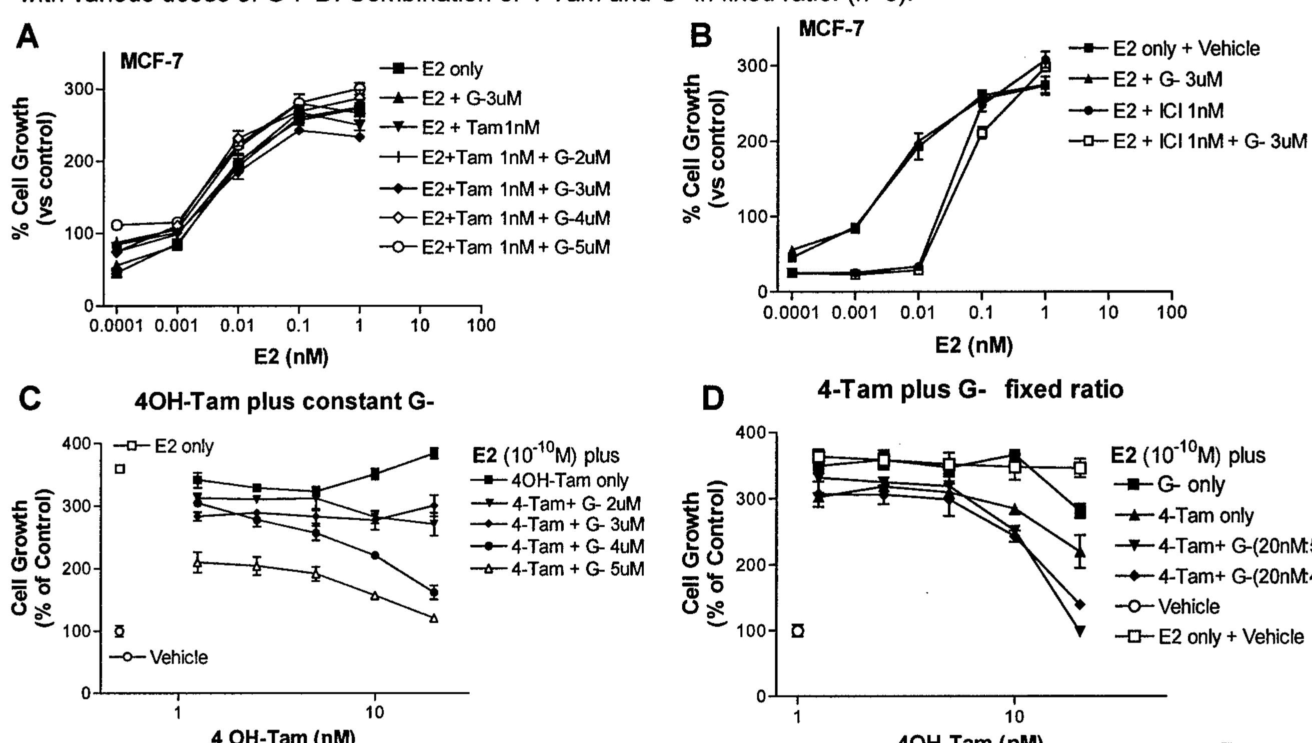
		IC50 (nM)			Fold sensitization (Dose reduction Index)	
		Chemo only	plus G(-)	plus G(+)	G(-)	G(+)
T47D	DOX	75.77	4.2	7.74	18.0	9.78
	Gem	8.601	0.4	9.20	21.3	0.93
	Taxol	2.367	<0.001	0.006	>20	
MCF7	DOX	175.3	<10	97.61	>17.5	1.79
	Gem	54.4	<3	29.11	>18	1.87
	Taxol	7.925	<1	3.33	>8	2.37

II.2.1.(c) Gossypol in combination with hormone therapy

Similarly, we tested the combination activity of gossypol with Tamoxifen (Tam) and Feslodex (ICI). MCF-7 and T47D cells were stripped with phenol red free IMEM, then plated in 96-well plates (3000 cell/well), treated with the drugs alone or in combination. 7-days later, the cell survival was determined by WST-1 cell counting kit (Roche). The combination Index (CI) and Dose Reduction Index (DRI) for each combination dose ratio was calculated as described above.

Using the optimal conditions for MCF-7 (E2 = 0.1nM, culture time 7 days, 3000 cell/well of MCF-7 cells) obtained from ***II.1.2.*** **(Figure 2)**, we carried out cytotoxicity assays of gossypol in combination with anti-

Figure 6. WST cytotoxicity assay of gossypol in combination of anti-estrogen agents in MCF-7 cells. **A**: Combination of 1nM Tamoxifen (Tam) with various doses of gossypol (G-). **B**: Combination of 1nM Feslodex (ICI) with various doses of gossypol (G-). **C**: Combination of 1nM 4-hydroxy-Tamoxifen (4OH-Tam or 4-Tam) with various doses of G-. **D**: Combination of 4-Tam and G- in fixed ratio. (n=3).



estrogen reagents, Tamoxifen (Tam), 4-hydroxy-Tamoxifen (4-Tam) or Feslodex (ICI). As shown in **Figure 6A and 6B**, the combination of gossypol with Tam or ICI did not shift the dose-response curves, indicating that no obvious drug-drug interactions were present with the conditions tested. We also tested 4-hydroxy-Tamoxifen, which is more potent than Tam *in vitro*. The combination of constant dose of gossypol with 4-Tam **(Figure 6C),** or combining gossypol and 4-Tam in fixed ratios **(Figure 6B**), all showed additive activity. No significant synergy was observed in the conditions tested. Similar results were obtained in T47D cells.

To investigate whether this observation is true for other Bcl-xL inhibitors, we also employed another small molecule Bcl-xL inhibitor, BL-106, which has a potent binding affinity to both Bcl-2 and Bcl-xL. Here again, we observed the similar results as with gossypol.

Taken together, with the experimental conditions tested so far, our data show that the small molecule Bcl-xL inhibitor gossypol can sensitize breast cancer cells to chemotherapy; however, no significant sensitization was observed with hormone therapy.

II.2.2. Small interfering RNA (siRNA) against Bcl-xL

Small interfering RNA (siRNA) against Bcl-xL was established. We designed the siRNA based on Bcl-xL mRNA and constructed psiBcl-xL, a vector-based siRNA, for establishing stable cancer cell clones with Bcl-xL knockdown. A siRNA specific to Firefly luciferase gene, psiLuc, was used as vector control.

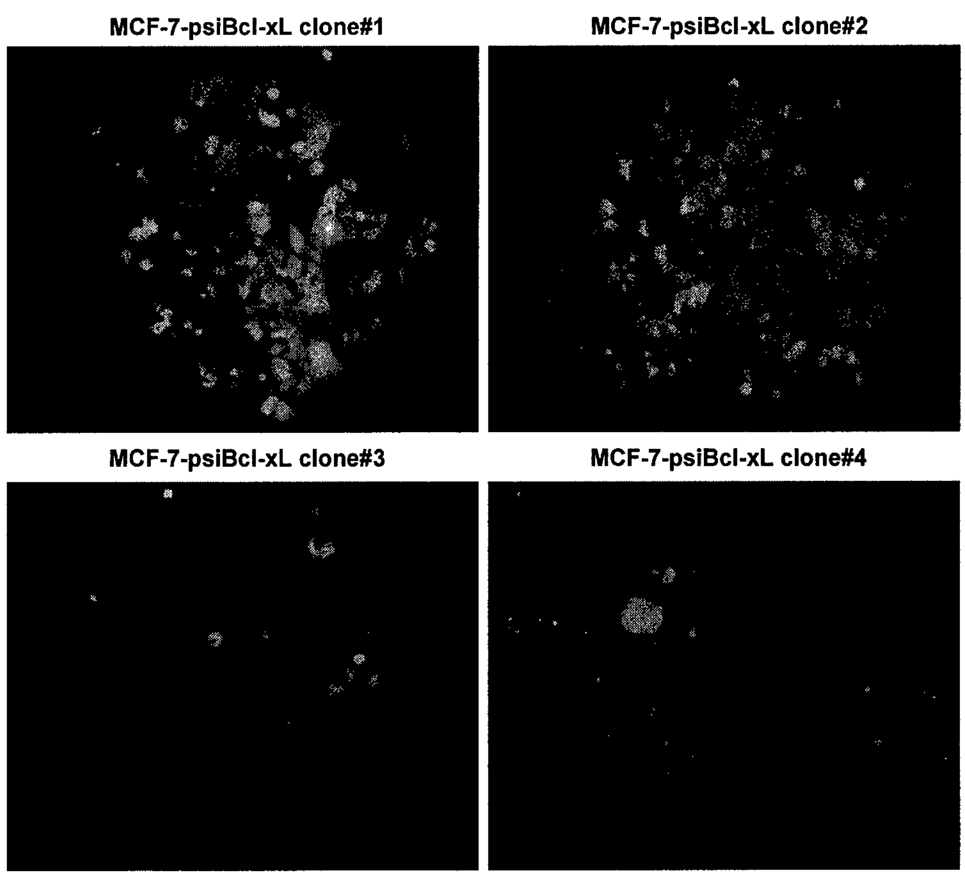
II.2.2.(a) Transfection of MCF-7 cells with Bcl-xL siRNA vector, psiBcl-xL

MCF-7 cells were transfected with psiBcl-xL as we previously described(5). In some experiments, FuGene 6 (Roche) was used due to low toxicity and high efficiency for plasmid transfection. Briefly, 60% confluent cells were transfected with 1-3 ug psiBcl-xL or psiLuc DNA each well in a 6-well plate (DNA:FuGene 6 ratio = 1ug:3ul). After 48 hr culture, the cells were collected and lysed for Western analysis, or trypsinized and replated into 6-well plates, and 50 – 800 ng/ml hygromycin (Invitrogen) was added for stable clone selection.

The transfection efficiency of FuGene 6 for MCF-7 was about 40% - 50%, as evidenced by the green fluorescence in the transfected cells, since psiBcl-xL has green fluorescence protein (GFP) gene to monitor the transfection. MCF-7 cells transfected with psiBcl-xL showed significant cell death (up to 40%).

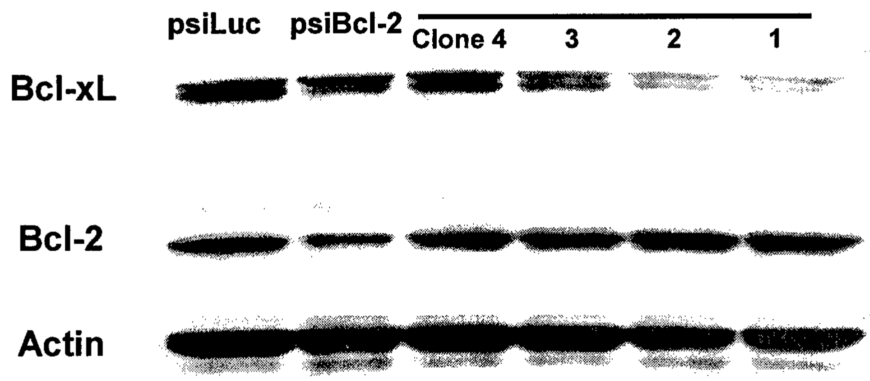
For stable clone selection, the MCF-7 cells were cultured in hygromycin selection media for two to

Figure 7. Fluorescent photos of MCF-7 psiBcl-xL stable clones. psiBcl-xL clones show different levels of green fluorescence protein (GFP) expression.



three weeks and 4 clones with strong green fluorescence were picked. **Figure 7** shows the fluorescent photos of MCF-7-psiBcl-xL stable clones. Note that the different MCF-7-psiBcl-xL clones have different levels of green fluorescence protein (GFP) expression. Theoretically, the level of GFP expression is proportional to the copy number of psiBcl-xL stably transfected in the cells, which is also corresponding to the level of Bcl-xL siRNA transcription from the plasmid psiBcl-xL. Indeed, as shown in **Figure 8**, the Western blot analysis of MCF-7 psiBcl-xL stable clones, the MCF-7-psiBcl-xL clone #1 and #2 showed >95% and >90% down-regulation of Bcl-xL gene expression, respectively, whereas no significant effects on Bcl-xS and Bcl-2. Thus, the clones #1 and #2 have the strongest down-regulation of Bcl-xL, while these two clones also have the strongest green fluorescence. Interestingly, the clone #3 showed >60% down-regulation of Bcl-xL and has moderate green fluorescence, while the clone #4 showed no significant Bcl-xL-downregulation together with poor green fluorescence.

Figure 8. Western blot analysis of MCF-7 psiBcl-xL stable clones. psiBcl-xL clone #1 and #2 showed >95% and >90% down-regulation of Bcl-xL gene expression whereas no significant effects on Bcl-xS and Bcl-2. The clone #3 showed >60% down-regulation.



II.2.2.(b) <u>siRNA-mediated down-regulation of Bcl-xL resulted in sensitization of MCF-7 cells to chemotherapy</u>

Overexpression of anti-apoptotic protein Bcl-xL renders cancer cells more resistance to chemotherapeutic agents. Down-regulation of Bcl-xL by psiBcl-xL will overcome this apoptosis-resistance, thus enhance the induction of cell death by chemotherapy. As shown in Figure 8, the MCF-7 psiBcl-xL Clone#1 showed 5-fold more sensitive than psiLuc clone to docetaxel (TXT)-induced cell death (Figure 8A). The Clone#1 also showed a moderate sensitization to CDDP, although to a less extent (Figure 8B). The Clone#1 is the clone that has the best Bcl-xL gene knockdown (>95%, Figure 7).

Taken together, transfection of MCF-7 cells with psiBcl-xL resulted in up to 90% -- 95% down-regulation of Bcl-xL protein. MCF-7 cells transfected with psiBcl-xL showed significant increase of cell death and/or apoptosis, slowed cell growth and increased sensitivity to chemotherapeutic agents. This tumor inhibitory activity of psiBcl-xL is related to the down-regulation of the intracellular level of Bcl-xL protein, by specific RNA-interference of Bcl-xL mRNA.

II.2.3. Pilot in vivo studies

II.2.3.(a) <u>Tumorigenecity of MCF-7 stable clones with Bcl-xL knock-down</u>

For each MCF-7-psiBcl-xL stable clones, 10 x 10^7 cells were inoculated in mammary fat pad of ovariectomized female nude mice (Ncr-nu/nu), after E2 pellets were put in. The tumorigenecity of these MCF-7 clones was summarized in **Table 3**. The Clone#1 did not show any sign of xenograft tumor growth 8 weeks after inoculation. The Clone #3 and #4 had only one tumor out of 6 inoculations. Interestingly, Clone #2 had 4 tumors out of 6 inoculations (Table 3), this appears to be consistent with the data shown in **Figure 8** that the Clone #2 cells were more resistant to chemotherapy especially CDDP. This Clone #2 may have other pro-growth/anti-death genes overexpressed to compensate for the down-regulated Bcl-xL for protection. We are currently doing more studies to characterize these clones for their molecular profiles, drug response, etc. These clones provide us with a useful tool to delineate the role of Bcl-xL in ER(+) breast cancer initiation, progression, and drug resistance.

Figure 9. Cytotoxicity assay of MCF-7 psiBcl-xL stable clones. MTT-based WST assay was carried out in 96-well plate. The stable clones of transfected MCF-7 cells were plated 3000 cell/well, treated with CDDP and docetaxel (TXT) for 4 days, add WST-1 as described. The results are plotted as viable cells (% of untreated control).

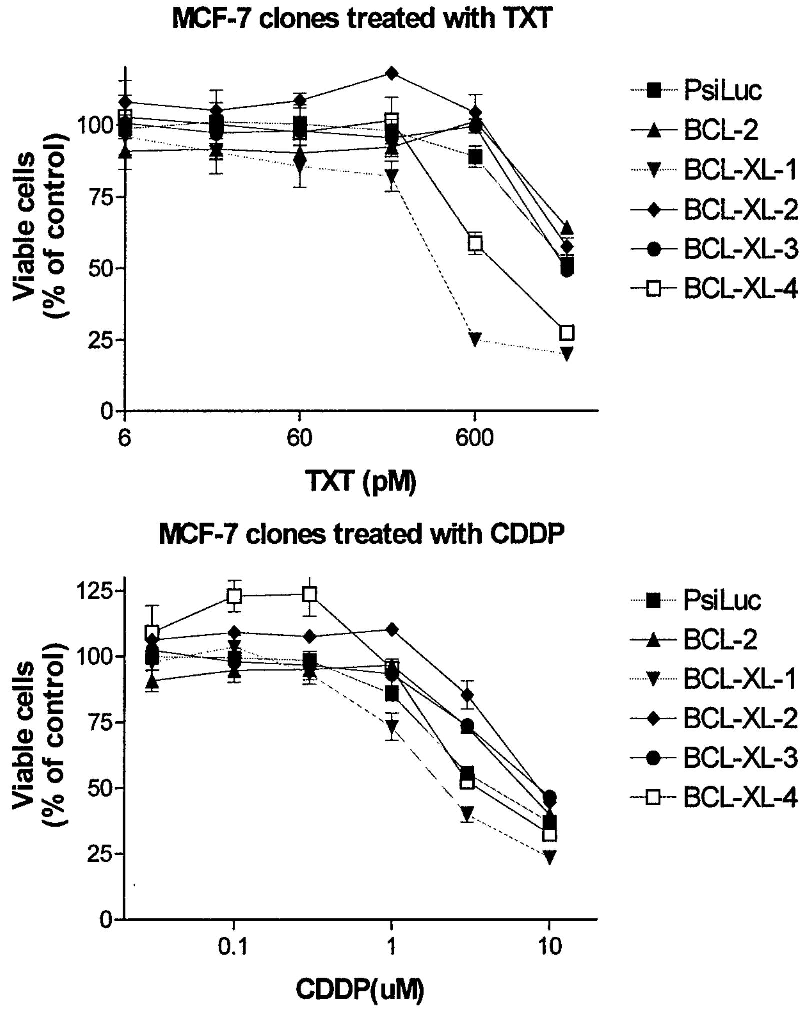


Table 2. Tumorigenecity of MCF-7-psiBcl-xL stable clones in nude mice (Day 19)

Mouse #	psiLuc	psiBcl-xL-1	psiBcl-xL-2	psiBcl-xL-3	psiBcl-xL-4
1	21.44	0	46.4	0	0
2	23.28	0	0	0	0
3	226.48	0	48.75	0	0
4	72.48	0	127.87	13.50	0
5	51.77	0	0	0	46.23
6	0	0	7.7175	0	0
Mean	65.91	0.00	38.46	2.25	7.70
SD	82.66	0	49.12	5.51	18.87

Values are tumor sizes (mm3).

II.2.3.(b) Proof-of-concept pilot *in vivo* study in MCF-7 tumor regrowth model, to see whether Bcl-xl inhibitor can delay or even prevent the regrowth (relapse) of resistant tumor

For MCF-7 tumor regrowth study, 10 x 10^7 cells were inoculated in mammary fat pad of ovariectomized female nude mice (Ncr-nu/nu), after E2 pellets (1.7 mg) were put in. 3-4 weeks later, when the tumors reach about 50 mm3 size, the E2 pellets were removed and Tamoxifen pellets (5 mg) were put in. Animal grouping: (a) positive control with new E2 pellet, (b) negative control with no E2 implant, (c) TAM only group with TAM pellet (5 mg) implant, (d) Gossypol only group, fed gossypol in drinking water, (e) Gossypol+TAM group with TAM implant, fed gossypol in drinking water. However, in the middle of experiment, the gossypol groups (Groups d and e) animals died in the second week of putting gossypol in drinking water, possibly due to toxicity. In pre-test for toxicity of gossypol, we have fed gossypol in drinking water to regular Balb/c mice for three weeks without significant toxicity, only minor body weight loss and the appearance of limited dehydration. It appears that the ovariectomized nude mice are more fragile and sensitive to gossypol. Further toxicity analysis is underway to determine the maximum tolerated dose (MTD) and the dose limiting toxicity (DLT) in nude mice with gossypol fed in drinking water.

III. Reportable outcomes:

1. One peer reviewed grant awarded in 2004:

Based on the data obtained funded in part from this Concept Award grant, we applied and obtained a Pilot Project award from NIH NCI SPORE program in University of Michigan, working on (-)-gossypol and its derivatives in radiosensitization of human prostate cancer.

SPORE 2 P50 CA69568-06A1 (Program P.I.: K. Pienta) 10/01/04 – 9/31/05
NIH/NCI Pilot project in SPORE in Prostate Cancer $90,000
Potent Bcl-2/Bcl-X_L-bispecific Small Molecule Inhibitor, (-) Gossypol, as Novel Molecular Targeted Therapy for Hormone-Refractory Prostate Cancer
The major goal of the proposal is to test the activity of Gossypol alone or in combination with radiotherapy for treatment of prostate cancer.
Role: Principal Investigator in this pilot project.

2. One Program Project (U19) grant applied in 2004 and likely to be funded:

In 2004, we applied a U19 Program Project grant which consists of three R01 type Laboratory Programs. The U19 grant scored **131**, percentile **2.5%**, likely to be funded. Some of the data was obtained from the Concept grant.

1 U19 CA113317 (P.I.: Shaomeng Wang) 05/01/2005 – 04/30/2010
NIH NCI U19 Program grant $814,294/year
Novel small-molecule inhibitors of Bcl-2/Bcl-xL proteins
The major goal of the proposal is to discover and design more potent small molecule based modulators of the functions of Bcl-2/Bcl-xl proteins
Role: **Co-leader** in Laboratory Program #3 (with budget $257.087/year)

3. Four abstracts funded from this grant were presented in international meetings. One abstract was selected for **Press Release** by the Organizing Committee in *EORTC-NCI-AACR Conference on "Molecular Targets and Cancer Therapeutics"* in Geneva, Switzerland, 2004. The PI, Dr. Xu, was interviewed by several press agencies during the meeting and there have been over 10 news reports published discussing the promising new therapy for prostate cancer funded by the PCRP grant. In the *International Conference on Tumor Progression and Therapeutic Resistance*, Dr. Xu was awarded the **2nd Prize of Poster Award.**

- Liang Xu, et al. Discovery and therapeutic potential of novel Bcl-2/Bcl-xL small-molecule inhibitors in human breast and prostate cancer. *International Conference on Tumor Progression and Therapeutic Resistance.* Philadelphia, PA, November 8-9, 2004. (Awarded **2nd Prize of Poster Award**).

- Liang Xu, et al. Radiosensitization of Human Prostate Cancer by Natural Polyphenol Inhibitor of Bcl-2/XL, (-)-Gossypol, Results in Tumor Regression. *EORTC-NCI-AACR Conference on "Molecular Targets and Cancer Therapeutics,"* Geneva, Switzerland, September 28-October 1, 2004. (Selected for **Press Release** by Organizing Committee).
- Liang Xu, et al. Gossypol(-), a Potent Small Molecule Inhibitor of Bcl-2/xl, Improves Response to Radiation Therapy and Results in Tumor Regression of Human Prostate Cancer. *The 95th American Association for Cancer Research Annual Meeting,* Orlando, Florida, March 27-31, 2004.
- Liang Xu, Dajun Yang, Shaomeng Wang, Wenhua Tang, Zaneta Nikolovska-Coleska, Hongpeng Liu, Xihan Wu, Min Ji, Jianyong Chen, Yan Ling, York Tomita, and Marc E. Lippman. Improved anti-tumor efficacy of chemotherapy by (-)Gossypol, a potent small molecule inhibitor of anti-apoptotic protein bcl-XL. *AACR-NCI-EORTC International Conference on "Molecular Targets and Cancer Therapeutics"*, Boston, MA, November 17-21, 2003

4. One investigational new drug (IND) application filed in 2004, pending FDA approval, on (-)-gossypol safety in human beings.

5. One provisional patent application filed with USPTO on 12/27/2004, title: The siRNA-based therapeutics targeting Bcl-xL.

IV. Conclusions:

The major goal of this Concept Award project is to investigate whether a small molecule inhibitor of Bcl-xL will be able to overcome the chemo- and endocrine-resistance in breast cancer. We have investigated the *in vitro* anti-tumor activity of (-)-gossypol, a potent small molecule inhibitor of Bcl-xL, and the potential synergistic effects of (-)-gossypol in combination with chemodrugs and Tamoxifen in breast cancer cell lines. (-)-gossypol showed potent anti-tumor activity to human breast cancer cell lines with high levels of Bcl-xL, but has only minimal effect on human normal breast epithelial cells with low Bcl-xL. (-)-gossypol potently enhanced growth inhibition by doxorubicin and docetaxel, currently used chemotherapeutic agents for breast cancer, both *in vitro* and *in vivo*. However, (-)-gossypol did not show significant enhancement of Tamoxifen or Feslodex activity in ER(+) breast cancer MCF-7 and T47D cells. Bcl-xL knockdown by siRNA abolished the tumorigenecity of MCF-7 cells, as well as sensitized the tumor cells to chemotherapy. The data support that Bcl-xL plays a critical role in breast cancer initiation, progression and chemoresistance, but its role in endocrine resistance remains to be further elucidated. The study provide us a solid foundation to develop (-)-gossypol as a novel molecular targeted therapy for the treatment of breast cancer with Bcl-xL overexpression.

V. References:

1. Zhang, M., Liu, H., Guo, R., Ling, Y., Wu, X., Li, B., Roller, P. P., Wang, S., and Yang, D. Molecular mechanism of gossypol-induced cell growth inhibition and cell death of HT-29 human colon carcinoma cells. Biochemical Pharmacology., *66:* 93-103, 2003.
2. Kitada, S., Leone, M., Sareth, S., Zhai, D., Reed, J. C., and Pellecchia, M. Discovery, characterization, and structure-activity relationships studies of proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins. Journal of Medicinal Chemistry., *46:* 4259-4264, 2003.
3. Chou, T. C., Motzer, R. J., Tong, Y., and Bosl, G. J. Computerized quantitation of synergism and antagonism of taxol, topotecan, and cisplatin against human teratocarcinoma cell growth: a

rational approach to clinical protocol design.[comment]. Journal of the National Cancer Institute., *86:* 1517-1524, 1994.

4. Chou, T. C., Tan, Q. H., and Sirotnak, F. M. Quantitation of the synergistic interaction of edatrexate and cisplatin in vitro. Cancer Chemotherapy & Pharmacology., *31:* 259-264, 1993.

5. Xu, L., Frederik, P., Pirollo, K. F., Tang, W. H., Rait, A., Xiang, L. M., Huang, W., Cruz, I., Yin, Y., and Chang, E. H. Self-assembly of a virus-mimicking nanostructure system for efficient tumor-targeted gene delivery. Human Gene Therapy., *13:* 469-481, 2002.

VI. Appendix

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